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TANDEM RECOMBINANT PLASMID CONSTRUCTION AS POSITIVE CONTROL FOR PIK3CA H1047R DETECTION BASED ON SYBR GREEN I qPCR

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ABSTRACT

PIK3CA H1047R mutation is found in breast cancer in high frequency and its detection could be applied as prognosis and predictive factor for trastuzumab therapy. qPCR is one of the simplest and robust method for PIK3CA H1047R detection. Provision of positive control for PIK3CA H1047R detection based on qPCR will support data analysis efficiently and avoid false negative result. In this research, we constructed a tandem recombinant plasmid (pGEM-tandPIK3CA) as positive control for Tm Shift SYBR Green I qPCR-based of PIK3CA H1047R detection system by ligating wild-type and PIK3CA H1047R fragments tandemly into pGEM-T Easy. The tandem plasmid was confirmed by restriction, DNA sequencing and qPCR. As a result, pGEM-tandPIK3CA has been successfully constructed and confirmed. Statistical analysis shows high repeatability and reproducibility with % CV of <25%. The main advantage of this tandem positive control is its ability to serve as positive control for both wild-type PIK3CA and PIK3CA H1047R simultaneously, therefore improving the efficiency of the detection system.

Keywords: Breast cancer, PIK3CA H1047R, qPCR, positive control

INTRODUCTION

Breast cancer is one of the most common type of cancer suffered by women and is found to be a leading cause of death among women, second only to cervical cancer [Muhartono *et al*., 2018]. Breast cancer is a heterogeneous disease at the molecular level regardless of the patient's race and geographic location [Bhargava *et al*., 2011]. In Indonesia, the prevalence of breast cancer reaches 0.5% of women population [Susianti *et al*. 2018]. One of the somatic mutations that is observed in approximately 30% of breast cancer, more recurrent in estrogen receptor (ER) positive breast cancer, is of PIK3CA [Miller, 2012]. PIK3CA is the gene encoding the p110-α catalytic subunit of phosphatidylinositol-3-kinase (PI3K), a family of lipid kinase involved in many vital cellular processes, such as cell growth, proliferation, differentiation, motility, adhesion, apoptosis, metabolism and survival [Ang *et al*., 2013, Li *et al*., 2016, Gkeka *et al*., 2014]. There are three hotspots of somatic mutations in PIK3CA that are most frequently (more than 80%) observed: the helical domain (E545K and E542K in exon 9) and the kinase domain (H1047R in exon 20) [Engelman *et al.*, 2008, Higgins *et al*., 2012]. Study showed that mutation in catalytic domain of PIK3CA is 5.3% higher than other PIK3CA mutations, and PIK3CA H1047R somatic mutation has the highest frequency within this domain [Stemke-Hale *et al*., 2012].

Several studies have shown that the high frequency of PIK3CA mutations and the investigations on these genetic mutation sites could bring remarkable clinical implications for diagnosis, prognosis and therapy of cancer [Karakas *et al*., 2006]. The oncogenic mutation of PIK3CA has been linked with transtuzumab resistance in cell culture and was associated with a poor prognosis after trastuzumab therapy [Berns *et al*., 2007]. Therefore, the necessity for an effective detection system of this mutation becomes increasingly important.

Currently, mutations can be detected by using numerous methods. DNA sequencing is one of the detection methods used to this day but limited by its low sensitivity (20-30%) [Li *et al*., 2016]. Other technologies with higher sensitivity such as next generation sequencing (NGS) and digital droplet PCR have been developed, but they are costly since it requires special and expensive equipment and trained personnel to be operated. Real-time polymerase chain reaction (real-time PCR) or also known as quantitative PCR (qPCR) is applied widely as a method to detect gene mutations. This method gives a sensitive and robust data at low cost and can be applied in several laboratory facilities [Alvarez-Garcia *et al*., 2018]. One of the approaches in applying qPCR is the use of SYBR Green I as fluorescent dye which is also inexpensive.

Although it is regarded as a simple, robust, low cost and sensitive method in gene detection, qPCR often still produce compromising results, such as generating false positive and false negative results [Niwa *et al*., 2007]. These false results could be due to various factors, one of which is the lack of adequate quality controls. Positive control is one of the quality controls used in qPCR method to assure the optimum PCR reaction and prevent the false negative results caused by inhibitor in the reaction, the lack of DNA polymerase activity, and the error of thermal cycler [Hoorfar *et al*., 2004, Majidzadeh *et al*., 2014]. Therefore, the presence of positive control is important in gene detection using qPCR method.

This current work aimed to construct a positive control for simultaneous detection method of PIK3CA WT and PIK3CA H1047R mutations on breast cancer based on Tm shift SYBR Green I qPCR assays that are efficient, inexpensive, easy and reproducible. Provision of simultaneous positive control for detection of wild-type PIK3CA and PIK3CA H1047R is important since both mutant and wild-type cells are present in the sample.

MATERIALS AND METHODS

DNA templates: DNA templates, PIK3CA Exon 20 wild-type and PIK3CA H1047R mutant gene sequence that were inserted into plasmid vector pGEM-T Easy (Promega, USA) separately, were obtained from Molecular Biology and Diagnostic Laboratory, Research Center for Biotechnology, LIPI, Cibinong-Indonesia. Each recombinant plasmid was separately transformed into competent *Escherichia coli* DH5α cells using heat shock method. The bacteria were cultured to produce multiple copies of the plasmid. Plasmid then extracted using High-Speed Plasmid Mini Kit (Geneaid, Taiwan) according to manufacturer's instruction and quantified for its concentration and purity using GeneQuant pro spectrophotometer (Amersham, UK).

Primers: Cloning primers were designed based on the sequences of PIK3CA human gene from GenBank of National Centre for Biotechnology Information (NCBI) with accession number: NG_ 012113.2. The primers were designed to amplify the 519 bp fragment of PIK3CA gene that covers exon 20 sequences and containing either mutations or not (Table 1). Meanwhile, genotyping of wild-type PIK3CA and PIK3CA H1047R based on Tm Shift SYBR Green I qPCR was performed using a patented primer (number P00201703489).

Table 1: Primers designed

Primer	Sequence (5' → 3')
PIK3CA-Exon 20-Fw	CATTTGCTCCAAACTGACCAAA
PIK3CA-Exon 20-BamHI-Fw	GGGCCGGATCCCATTTGCTCCAAACTGACCAAA*
PIK3CA-Exon 20-BamHI-Rev	CCCGGGGATCCTTTTGAAATGAACTAGTTTAAGTGC*

*BamHI restriction recognition site (GGATCC) are underlined

Optimization of the annealing temperature: Annealing temperature was optimized using a gradient PCR method at temperature of 55–62°C and using 0.5µL of recombinant plasmid pGEM-T *Easy*–PIK3CA H1047R (pGEM-PIK3CAH1047R) as template. PCR was performed using 5µL DreamTaq Green PCR Master Mix (2×) (Thermo Scientific, US), consisting of primer PIK3CA-Exon 20-Fw (0.5µL) and PIK3CA-Exon 20-BamHI-reverse (0.5µL) and MQ to reach total volume of 10µL. The PCR condition was set as follows: predenaturation at 95°C for 5 min, followed by 35 denaturation cycles at 95°C for 30 s, annealing at 55-62°C for 30 s, extension at 72°C for 1 min, and post-cycling at 72°C for 5 min. PCR products were electrophoresed on 1% agarose gel for 25 minutes at constant voltage of 110 volt. Gel was stained with ethidium bromide (EtBr) and visualized under UV light using UV transilluminator.

Cloning of the tandem recombinant plasmid: The tandem recombinant plasmid was constructed as Fig. 1. Amplification of PIK3CA H1047R (519 bp) gene was conducted using primer pairs, which introduced BamHI site at 3' on the resulting amplicon. The total reaction volume was 12.5µL, consisting of 12.5µL of PCR Master Mix (2×) (Thermo Fisher Scientific, US), 0.1µL of each primer, 0.1µL of template (pGEM-PIK3CAH1047R) and MQ. PCR condition was set according to optimum annealing temperature. The resulting amplicon from PCR was confirmed using eectrophoresis on 1% agarose gel. The PCR product was purified using kit Wizard® SV Gel and PCR Clean-Up System (Promega, USA) according to manufacturer's manual and electrophoresed on 1% agarose gel. The purified product was ligated into vector plasmid pGEM-T *Easy* using T4 DNA ligase (Promega, USA) according to the manual instruction. The recombinant plasmid (pGEM-PIK3CAH1047R*Bam*) was cloned by using heat shock transformation method into competent DH5α cells, then confirmed by the colony PCR and electrophoresed on 1% agarose gel. The recombinant plasmid pGEM-PIK3CAH1047R*Bam* was extracted using High-Speed Plasmid Mini Kit (Geneaid, Taiwan) according to the protocol, and

the concentration was determined using spectrophotometer. To further insert another gene tandemly, restriction of pGEM-PIK3CAH1047R*Bam* was performed using BamHI restriction enzyme (Thermo Fisher Scientific, USA) in a reaction consisting of 1µL 10× buffer BamHI, 5µL recombinant plasmid, 0.5µL BamHI and nuclease-free water until total volume of 10µL. The incubation was carried out at 37oC for 14 hours. The restriction product was confirmed on 1% agarose gel. The restricted pGEM-PIK3CAH1047R*Bam* was dephosphorylated using Calf Intestinal Alkaline Phosphatase (CIAP; Promega, USA) according to manual instruction, purified using alcohol precipitation method and confirmed on 1% agarose gel.

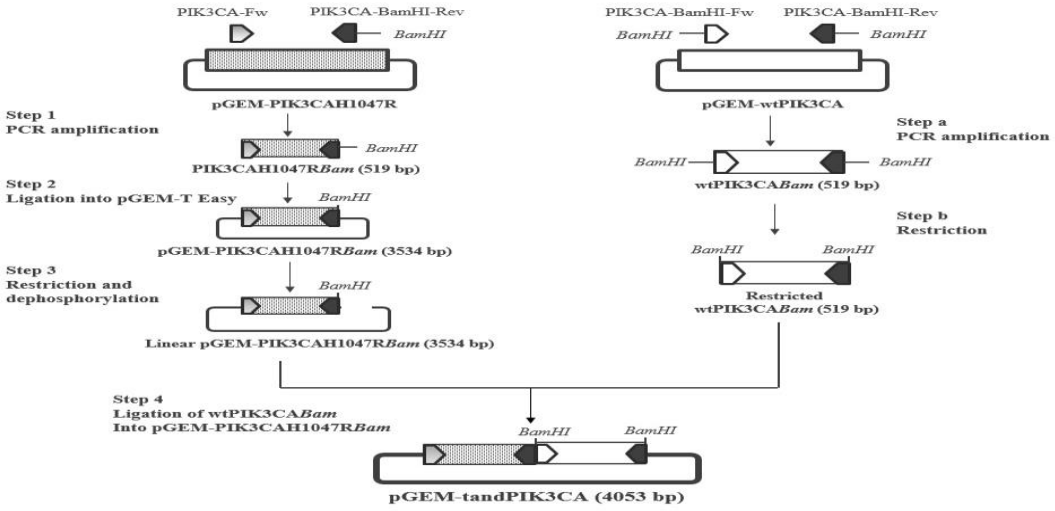


Fig. 1: Plasmid construction

The wild-type PIK3CA fragment previously introduced with BamHI at 5' and 3' (step a, Fig.1) was restricted (step b, Fig.1) and ligated tandemly on the downstream of pGEM-PIK3CAH1047R*Bam* (step 4, Fig. 1). The ligation was performed using ligase enzyme (Takara, US), and incubation was carried out at 4 °C overnight. The ligation product (10µL), i.e recombinant plasmid pGEM-T *Easy* with PIK3CAH1047R*Bam* and wtPIK3CA*Bam* (pGEM-tandPIK3CA), was transformed into 70µL of competent cells DH5α using heat shock method. The plasmid was extracted using kit and confirmed using electrophoresis on 1% agarose gel. The plasmid concentration from the extraction process was determined using spectrophotometer.

Confirmation of tandem positive control: The tandem recombinant plasmid was confirmed using three methods: BamHI restriction digest analysis, DNA sequencing and qPCR. 5µL of pGEM-tanPIK3CA was restricted using *Bam*HI in a total volume of 10µL consisting of 0.5µL *Bam*HI, 1µL 10× buffer *Bam*HI and nuclease-free water. The incubation was performed at 37°C for 14 h. The restriction product was electrophoresed on 1% agarose gel.

DNA sequencing was also performed to confirm the resulting positive control. DNA was sequenced by First Base in Malaysia, while the resulting DNA sequencing was analyzed using software BioEdit v7.2.0.

qPCR experiment was carried out using tandem recombinant plasmid of pGEM-tanPIK3CA as DNA template. PCR reaction was based on the patent using BioRad CFX Manager version 3.

Statistical analysis: Reproducibility and repeatability of the resulting positive control was assessed by calculating variance coefficient (%CV) from melting temperature. The smaller %CV (≤ 25%) indicated better reproducibility and repeatability [Broeders *et al*., 2014].

RESULTS

Optimization of annealing temperature: Prior to construction of tandem plasmid, the annealing temperature was optimized to obtain the single band of targeted DNA fragment. This enables to perform purification for cloning experiment more easily. The optimization of annealing temperature using gradient PCR resulted in a clear band at 57 °C, with size of 519 bp in conjunction with the targeted size. Therefore, annealing temperature of 57°C was set as the optimum temperature and applied in the further PCR reaction for target gene cloning. The single band of PCR product could make the further purification easier.

Cloning of PIK3CA H1047R: The fragments of PIK3CAH1047R*Bam* (519 bp) were inserted into vector plasmids pGEM-T easy (3015 bp), yielding

recombinant plasmids pGEM-PIK3CAH1047R *Bam* with the length of 3534 bp *(Fig. 2).*

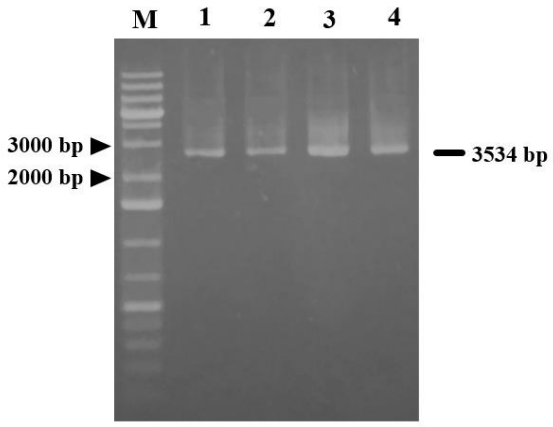


Fig. 2: pGEM-PIK3CAH1047R*Bam* recombinant plasmid (3534 bp), M: marker, 1-4: pGEM-PIK3CAH1047R*Bam* bands

BamHI restriction digestion and phosphatase treatment of pGEM-PIK3CAH1047R*Bam:* PIK-3CA H1047R were amplified with primers that introduced one BamHI digestion site and then inserted into plasmid vector pGEM-T easy. 5µL recombinant plasmid pGEM-PIK3CAH1047R were digested with 0.5µL BamHI restriction enzyme, therefore linearized the plasmid. Digested recombinant plasmid then dephosphorylated to prevent self-ligation. Digestion and dephosphorylation results were later confirmed on 1% agarose gel, that showed a single band with the length of 3534 bp (data not shown).

PCR amplification and BamHI digestion of wild-type PIK3CA fragment: Wild-type PIK3-CA was amplified to insert the PIK3CA WT gene tandemly on the downstream site of PIK3CAH-1047R*Bam* that was inserted in previous step. Wild-type PIK3CA fragment (wtPIK3CA*Bam*) possessed two BamHI restriction sites introduced by primer used in the PCR amplification. The amplification of PIK3CA wild-type fragment (3µL) successfully resulted in a clear single band, specifically at size of about 519 bp (Fig. 3A)

Prior to insertion, wild-type PIK3CA fragment was restricted using BamHI and qualitatively confirmed using electrophoresis. The restricted fragment was then compared to unrestricted fragment (Fig. 3B)

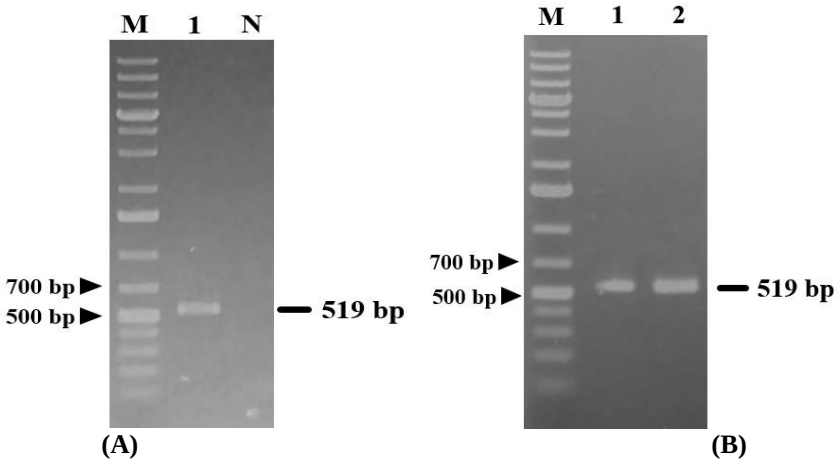


Fig. 3: Amplified wtPIK3CA*Bam* fragment (A), M: marker, 1: wtPIK3CA*Bam* fragment band (519 bp), N: NTC (non-template control); restriction result of wtPIK3CA*Bam* (B), M: marker, 1: unrestricted fragment, and 2: restricted fragment.

Ligation of wild-type PIK3CA fragment (wtPI-K3CA*Bam*) into pGEM-PIK3CAH1047 R*Bam:* The resulting digested wtPIK3CA*Bam* fragment was ligated into recombinant plasmid pGEM-H10 47R*Bam* and transformed using heat shock method into *E. coli* DH5α. The transformation resulted in 10 white colonies that indicated the recombinant plasmid. The targeted recombinant plasmid screening was carried out using 10 white colonies. The extraction of those colonies showed, based on the size of plasmid pattern, two plasmids extracted from colony 7 and 9 presumably contained the desirable inserted gene (data not shown). To confirm this, three assays were prepared, namely restriction using BamHI, PCR sequencing, and qPCR.

Confirmation of tandem PIK3CA: After 14 hrs incubation on 37°C, 10µL digestion result was confirmed by electrophoresis on 1% agarose gel. There were two bands observed, 3534-bp length of pGEM-PIK3CAH1047R*Bam* and 519-bp length of wtPIK3CA*Bam* fragment (Fig. 4). This result confirmed the constructed tandem plasmid.

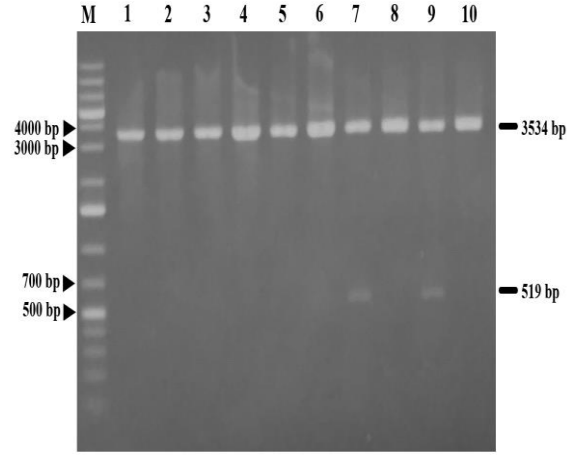
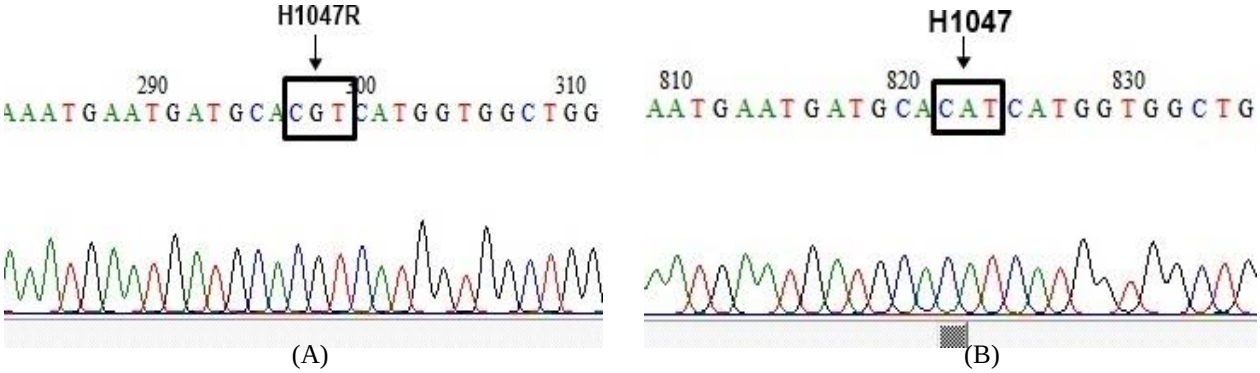


Fig. 4: Restriction result of the tandem recombinant plasmid; M: marker, 1-10: restricted pGEM-tand-PIK3CA

Confirmation by direct sequencing was also done and we observed the sequence of vector plasmid that contain both wild-type PIK3CA and PIK3CA H1047R (Fig. 5.)

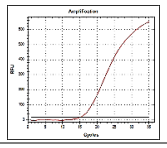
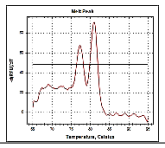
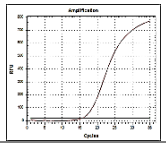
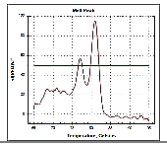
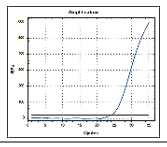
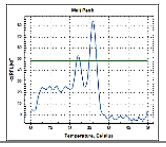
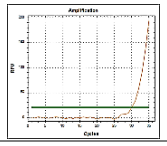
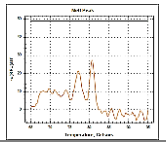
Confirmation by qPCR was done by observing the melting curve data of diluted plasmid (Table 2).



(A) (B)

Fig. 5: DNA sequence of the tandem recombinant plasmid PIK3CA H1047R (A), PIK3CA wild-type (B).

Table 2: Melting curve pattern of pGEM-tandPIK3CA with concentration of 1-0.0001 ng

Concentration (ng)	Ct	Melting curve pattern
1		
0,1		
0,001		
0,0001		

According to melting curve on qPCR, two peaks were found, which indicate presence of amplicon wild-type PIK3CA and PIK3CA mutant (H1047R).

Repeatability and reproducibility: The coefficient of variation of intra and inter-assay were found to be less than 25% (Table 3).

Table 3: Coefficient of variation (CV) value of intra-assay (repeatability) and inter-Assay (reproducibility)

Observer	Wild-type Tm value	Mean	SD	CV (%)	Mutant Tm value	Mean	SD	CV (%)
1	77.00	77.07	0.09	0.12	80.60	80.80	0.16	0.20
	77.00				81.00			
	77.20				80.80			
2	77.00	77.07	0.09	0.12	81.00	80.93	0.09	0.12
	77.20				80.80			
	77.00				81.00			

DISCUSSION

qPCR technologies had been used in some research for detection of low abundance somatic mutation since it is a method with high sensitivity, specificity and selectivity. However, there are still possibilities of false-negative results obtained by this method. In this study, we developed a new exogenous positive control for qPCR-based PIK3-CA H1047R detection system to assure an acceptable confidence in qPCR results and to distinguish true negative from false-negative results.

Plasmid DNA recombination is one of crucial molecular biology technique widely used in biotechnology for many research purpose [Hou *et al*., 2016]. In this research, we constructed recombinant plasmids to serve as positive control in PIK-3CA H1047R detection system. This recombinant plasmid contained two gene sequences, wild-type PIK3CA and PIK3CA H1047R, that inserted tandemly. Therefore, its main advantage is that it can be used as positive control for the detection of both wild-type and mutant PIK3CA (H1047R). Efficient detection system can be obtained by using this tandem positive control in genotyping qPCR since it can shorten operational time and cost of the system. We developed this positive control that able to detect wild-type PIK3CA and PIK3CA H1047R simultaneously because somatic mutations (such as PIK3CA H1047R) usually present in a very low abundance within wild-type sequences [Morlan *et al*., 2009]. To our best knowledge, this research is the first attempt to construct a tandem positive control for the detection of PIK3CA H1047R using Tm Shift SYBR Green I qPCR method.

Ligation and restriction using DNA ligase and restriction endonuclease enzymes, respectively, are the most common methods to construct recombinant DNA [Wang *et* al., 2018]. To construct more than one inserts into a plasmid vector, we depend on the ends of the fragments ligated. In this research we only performed a single digestion using only one restriction enzyme, BamHI, which provided sticky ends to both of the inserts in the recombinant plasmid. Using only one restriction enzyme to obtained proper sticky ends in fragments ligated for the vectors to close properly is cost effective and easier to execute in daily laboratory routine.

Confirmation using three methods (restriction digestion, sequencing and Tm Shift SYBR Green I qPCR) showed consistent results that the tandem positive control had been successfully constructed. The results confirmed by qPCR assay emphasized that tandem plasmid was well prepared. Using SYBR Green I as intercalating dye generates melt curve as it releases at the end of qPCR assay [Germer and Higuchi, 1999]. In this study, we confirmed the developed tandem positive control using Tm Shift SYBR Green qPCR methods that can differentiate PCR products based on the sequence, length and the content of GC in the product [Ririe *et al*., 1997]. Melting curve shows two peaks with shifting of melting temperature (Tm) between wild-type PIK3CA and PIK3CA H105-47R sequence, thus confirming the two gene sequence inserted in the plasmid vector. Background, NTC and Ct value were under threshold line, indicating that the result was not interfered. We too found that melting temperature in this experiment was similar to previous research. Using developed method, PCR reaction was capable of recognizing the targeted amplicon signal specifically. This showed that the constructed positive control could be easily applied, since it has a high degree of specificity to the target.

In detecting the existence of particular DNA sequence, or qualitative PCR, the data about sensitivity of the assay is necessary [Bustin *et al*., 2009]. Tm profile showed a high degree of similarity and consistency on the sample used ranging from 1 ng to 0.001 ng of prepared positive control. Up until concentration of 0.001 ng, the melting curve exhibited consistent value, while at concentration of 0.0001 ng, the resulting plasmid was unable to amplify as indicated by much lower Ct value and melting curve position under the threshold line. This suggests that the sensitivity of control plasmid was 0.001 ng in concentration. Further, statistical analysis result showed that % CV of both intra and inter-assay were less than 5%, which indicated desirable reproducibility and

repeatability. All these results showed the reliability of the constructed tandem plasmid.

In conclusion, in this research, an efficient, easy to produce, cost-effective and reproducible control positive for Tm Shift SYBR Green I qPCR-based detection of PIK3CA H1047R has been successfully developed.

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